Tissue Expression and Pharmacological In Vitro Analyses of mTOR and SSTR Pathways in Adrenocortical Carcinoma

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EFFECTS OF COMBINED EVEROLIMUS AND PASIREOTIDE WITH MITOTANE TREATMENT IN ADRENOCORTICAL CARCINOMA CELL LINES.

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ABSTRACT

New therapies for advanced adrenocortical carcinoma (ACC) are urgently needed, as the majority of the patients experience a rapid and inexorable progression despite surgery and adjuvant mitotane. *In vitro* data suggest that somatostatin receptors (SSTR) and mTOR pathway might represent reasonable targets for novel therapies, being involved in functionality and growth of adrenocortical cancer (ACC) cells. However, *in vitro* analysis of combination treatments targeting both mTOR and SSTR as compared to mitotane are poorly explored in ACC. This study aimed to investigate *in vitro* the effects on cell growth of pasireotide, everolimus and mitotane, alone or combined, in the two ACC cell lines H295R and SW13 (mitotane sensitive and resistant, respectively). Moreover, the tissue expression of mTOR pathway molecules and SSTR (types 1-5) was assessed in 58 ACC. In both cell lines, only everolimus induced a significant inhibition of cell growth. Conversely the combinations among mitotane, pasireotide and everolimus produced antagonistic effects on mitotane-induced growth inhibition on H295R cell line. An heterogeneous profile of mTOR-related molecules and SSTR expression was observed in ACC samples, being the mTOR pathway found activated in approximately 30% of cases. In conclusion, our data suggest caution in designing combinations of mitotane with other drugs potentially active in ACC, such as mTOR inhibitors or somatostatin analogs.

Keywords: adrenocortical carcinoma cell lines, mitotane, target therapy, everolimus, pasireotide, mTOR, somatostatin receptor

INTRODUCTION

Adrenocortical carcinoma (ACC) is a rare malignancy, which has an aggressive biological behavior and a dismal prognosis, with a 5-year survival limited to 16-44% [1, 2]. Surgical resection is the only curative therapy, and is often followed by adjuvant mitotane to increase the chance of cure [3, 4]. However, high recurrence rates are observed even in early-stage patients. Therapeutic options for disseminated disease are associated with adverse effects and do not clearly improve survival [2]. Mitotane remains the only FDA-approved drug, with reported response rates (based on uncontrolled trials and small case series) of 21%, when used as a single agent [2]. As for cytotoxic chemotherapy, the effect is relatively limited. Recently, in a phase III randomized prospective trial the regimen etoposide, doxorubicin and cisplatin combined with mitotane determined a longer progression-free survival with a 23.2% response rate, but clear-cut benefit on overall survival were not observed [5]. As the majority of the patients experience a rapid and inexorable progression, new therapies for advanced ACC are urgently needed. The most common molecular event in ACC is the overexpression of insulin-like growth factor receptor 2 (IGFR-2), detected in up to 90% of tumours [6, 7, 8, 9]. In a recent phase III study on advanced or metastatic ACC patients, [10] linsitinib, an inhibitor of both IGF-1R and insulin receptor, did not increase overall survival. However, the IGF-1R pathway is reported to synergize with several other signaling pathways, including mammalian target of rapamycin (mTOR) pathway. In this regard, a partial response with prolonged survival was reported [11] in a phase I clinical trial combining the anti-IGF-1R antibody cixutumumab with the mTOR inhibitor temsirolimus. Conversely, the administration of everolimus to advanced ACC patients in combination or previously treated with mitotane resulted in limited clinical improvement [12]. In ACC cell lines, an antiproliferative role has been documented with the mTOR inhibitors sirolimus [13], temsirolimus [14] and everolimus [15, 16]. Among other drug targetable molecules documented in adrenocortical tumors, there are

somatostatin receptors (SSTR) [17, 18, 19]. The multi-ligand somatostatin analog pasireotide was demonstrated to inhibit hormonal secretion in ACC cell lines without effects on cell proliferation [19]. Moreover, pasireotide has been considered an effective treatment of Cushing's disease since it inhibits ACTH and corticosterone release both *in vitro* and *in vivo* [20, 21, 22]. *In vitro* experiments on neuroendocrine tumor cell lines indicated that somatostatin analogs enhanced the antiproliferative action of rapamycin probably acting in synergy on the PI3K/AKT/mTOR signaling pathway [23, 24]. Finally, several clinical trials showed an additional therapeutic value in combining a somatostatin analog with everolimus in advanced neuroendocrine tumors [25, 26].

To the best of our knowledge, in ACC cell lines, two reports only addressed the issue of associated treatments, combining sirolimus and mitotane [13] and everolimus and sorafenib [16], showing an additive and a synergic antiproliferative effect, respectively.

Therefore, the aim of the present study was to further explore potential combination treatment strategies through the evaluation of the reciprocal effects of everolimus, pasireotide and mitotane in two ACC cell lines (the mitotane sensitive H295R and the mitotane resistant SW13). The expression levels of potential targets of mTOR inhibitors and somatostatin analogs in a series of resected ACC were also assessed.

MATERIAL AND METHODS

Cell culture and chemical reagents - NCI-H295R and SW-13 ACC cell lines were supplied from the American Type Culture Collection (LGC Standards s.r.l., Sesto San Giovanni, Milan, Italy). The H295R cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient mixture (DMEM/F12) (Sigma-Aldrich, St. Louis, USA) supplemented with 2 mmol/L L-glutamine, penicillin (25 units/mL), and streptomycin (25 mg/mL, all from Sigma-Aldrich) and 2.5% of Nu-Serum (BD Biosciences, San Jose, CA) and enriched with 1% di ITS+Premix (BD Biosciences,

San Jose, CA). The SW-13 cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Biowest, Nuaillè, France) and 2 mmol/L L-glutamine, penicillin (25 units/mL), and streptomycin (25 mg/mL, all from Sigma-Aldrich). o,p'DDD, (Sigma-Aldrich, St. Louis, USA) and everolimus (Sequoia Research, Pangbourne, UK) were dissolved in 100% methanol (Sigma-Aldrich, St. Louis, USA). Pasireotide was a generous gift from Novartis and was dissolved in sterile distilled water.

Treatment and cell viability assay - SW13 and H295R cell lines were seeded into 96-well plates in triplicates and treated with pasireotide and mitotane or everolimus, either alone or in combination, for 72h (from 10 nM to 10^3 nM for each drug). The combination between different drugs was performed at fixed concentration ratios (1:1). After incubation, Cell Proliferation Reagent WST-1 (Roche Applied Science, Germany) was added to each well in order to measure cell proliferation, following the supplied protocol. Cell viability ratios were calculated using the sigmoid inhibition model (GraphPad PRISM 5). Drug interaction between pasireotide, mitotane and everolimus was assessed using the combination index (CI), where CI < 1, CI = 1, and CI > 1 indicate synergistic, additive and antagonistic effects, respectively. CI was determined by IC50 of each drugs and the combination with drugs [27].

SSTR and mTOR mRNA expression evaluation with Real Time PCR - H295R and SW-13 cell lines were cultured into six-well plates and treated with 250nM of everolimus, mitotane and pasireotide, alone or in combination for 72 hours. Total RNA was extracted using Qiazol Reagent (Qiagen, West Sussex, UK). Complementary DNA was generated using M-MLVT RT (200U/µl) (Invitrogen, Invitrogen Corporation, USA) and oligodT primers (500µl/ml) (Invitrogen, Invitrogen Corporation, USA) from 1µg of total RNA according to standard protocols. Relative cDNA

quantification for somatostatin receptors or mTOR and an internal reference gene (β-actin) was done in duplicate using a fluorescence-based real-time detection method (ABI PRISM7900 Sequence Detection System - TaqMan; Applied Biosystems, Foster City, CA, USA) using pre-designed primers and probes (SSTR types from Life Technologies and mTOR and β-actin from Roche Applied Sciences). To analyze target gene expression in treated cells, mRNA levels were normalized to the housekeeping gene β-actin, then ΔΔCt calculation was performed and the corresponding values were expressed as $2^{-\Delta\Delta Ct}$.

Tissue collection – 58 adrenocortical carcinomas diagnosed according to the Weiss Score [28] (including six myxoid and nine oncocytic subtypes) were retrieved from the pathology files of the University of Turin at San Luigi Hospital. The nine oncocytic tumors were also reclassified according to the Lin-Weiss-Bisceglia scheme [29], and four of them proved to be of uncertain malignant potential according to this more appropriate score for oncocytic neoplasms. These cases were incorporated into two tissue microarrays, assembled as previously described [30]. The majority of these patients were treated at our Institution, which serves as a referral center for adrenocortical carcinoma therapy in Italy. Clinical and pathological data of these patients are summarized in **Table 1**. The study received ethical approval from the local Review Board of our Institution.

Immunohistochemistry – Five µm thick paraffin sections serial to those used for conventional hematoxylin and eosin staining were obtained for immunohistochemical reactions from the TMAs. The following antibodies were employed: p-mTOR (Ser 2448, rabbit monoclonal, clone 49F9, diluted 1/100; Cell Signaling, Beverly, MA, USA), p-p70S6K (Thr 389, mouse monoclonal, clone 1A5, diluted 1/400; Cell Signaling, Beverly, MA, USA), p-Akt (Ser473, rabbit polyclonal, diluted 1/40; Cell

Signaling, Beverly, MA, USA), p-4EBP1 (Thr 37/46, rabbit polyclonal, diluted 1/300; Cell Signaling), p-Raptor (Ser792, rabbit polyclonal, diluted 1/100; Cell Signaling), SSTR1 (mouse monoclonal, clone N35B 15E9, diluted 1/1000, supplied by Novartis Pharma), SSTR2 (rabbit monoclonal, clone UMB1, diluted 1/1000; Epitomics, Burlingame, CA, USA), SSTR3 (mouse monoclonal, clone N37A QS5G8A12, diluted 1/700, supplied by Novartis Pharma), SSTR4 (mouse monoclonal, clone N55 19E7-F7, diluted 1/600, supplied by Novartis Pharma), SSTR5 (mouse monoclonal, clone N38 17D10 2/38, diluted 1/500, supplied by Novartis Pharma).

Staining interpretation and scoring system – All slides were analyzed by two of us (ED, MV) and all samples were considered adequate for all antibodies in the presence of at least two suitable cores after sectioning and staining procedures. Staining was assessed for all but one antibodies, using a binary scoring system based on the evaluation of cytoplasmic/membrane staining: score 0= no staining, score 1= positive staining.

Statistical analysis - Reciprocal correlations among the different antibodies expression levels were made using a two-tailed Spearman's test. Staining patterns for each antibody tested were compared to clinical and pathological characteristics using Chi-square or Student's t tests, as appropriate. The prognostic impact of the different antibody expression levels was tested in univariate overall survival analysis using the Kaplan–Meier product limit estimate of survival distribution and the Log Rank test. All parameters with a significant impact on survival at univariate analysis were included in multivariate analysis using the Cox proportional hazard model. Statistical significance was set at $p<0.05$. All tests were performed using GraphPad Prism version 6.0 and IBM SPSS Statistics Version 20.

RESULTS

Everolimus, but not pasireotide, is active as cytotoxic agent in adrenocortical cancer cells

Everolimus induced a dose-dependent decrease of cell viability in the two adrenal cancer cell lines (**Figure 1**), more intense in SW-13 with IC50 values of 0.52 nM (standard error ± 0.55) (at 72 h of treatment) rather than in H295R cells with IC50 values of 3.23 nM (standard error ± 1.58) (at 72 h of treatment). Conversely, pasireotide had no evident effect on cell viability when both ACC cell lines were treated using increasing doses of the drug (IC50 values of 16.45±2.56 μM in SW13 cells and 5.16±0.85 in H295R cells).

Different effects of pasireotide, everolimus and mitotane combinations in mitotane-sensitive and insensitive adrenocortical cancer cells

We confirmed the previously demonstrated [31] drug-responsiveness profiles of the two cell lines, as, after a 72 hour treatment, mitotane produced poor cytotoxic effects in SW13 cell lines, while the viability of H295R cells was impaired. In the association of mitotane with everolimus, mitotane blocked everolimus activity in both SW-13 and H295R cell lines, independently of their responsiveness to mitotane, with a combination index (CI=1.38±0.38 and 4.56±3.13) indicating an antagonistic effect of the two drugs (**Figure 1A and 1D**). The association of mitotane and pasireotide determined opposing effects in the two cell lines. In fact, in mitotane insensitive SW-13 cells, the combination index (CI=0.22±0.15) indicated a synergistic anti-proliferative effect of mitotane and pasireotide. Conversely, the same combination produced an antagonistic effect on cell growth (CI=3.11±2.13) in mitotane-sensitive H295R cells (**Figure 1C and 1F**). Similarly, the combination of pasireotide and everolimus determined a synergistic cytotoxic effect (CI=0.69±0.25)

in SW-13 cells and an antagonistic effect (CI=2.55±1.60) on cell growth in H295R cells (**Figure 1B and 1E**).

Combined treatments of pasireotide, everolimus and mitotane differentially modulate SSTRs and of mTOR gene expression in adrenocortical cancer cells

At basal level, H295R and SW13 cell lines showed a very low expression of all SSTRs (data not shown). Pasireotide administration, alone or in combination with mitotane or everolimus, induced a modulation of gene expression of all SSTRs in H295R cells. An up-regulation of all SSTR types was found with the association pasireotide/mitotane, but a two-fold decrease of only SSTR2 levels was observed with the pasireotide/everolimus treatment. Conversely, SSTR gene expression levels did not vary in SW13 lines treated with the same combinations (**Figure 2**). Concerning mTOR gene expression, the treatment with everolimus, alone and in combination with mitotane or pasireotide, did not determine any change of the expression levels.

mTOR pathway molecules and SSTRs are heterogeneously expressed in ACC tissues and correlate with clinical and pathological parameters and survival

Overall, using immunohistochemistry, p-mTOR expression was observed in 18 (32%) cases, with a moderate or strong cytoplasmic reactivity (**Figure 3, A-E**), while the other downstream molecules were variably expressed in the cytoplasm of tumor cells (range 29-59%). SSTRs were variably expressed in 29% (SSTR2) up to 83% (SSTR4) of cases, with a membrane or cytoplasmic reactivity (**Figure 3, F-L**) (**Table 2**). Among the mTOR pathway molecules, a positive correlation was found only between p-p70S6K and p-4EBP (r= 0.4664, p=0.0002), while among SSTRs, only SSTR1 and SSTR3 were reciprocally correlated (r= 0.3132, p=0.0177). p-AKT was positive in younger patients

(Chi-square test, p=0.044), conversely SSTR1 and SSTR3 were positive in the older patient group (Chi-square test, p=0.029 and p=0.007 for SSTR1 and SSTR3, respectively).

p-mTOR and SSTR3 expression was significantly different in oncocytic ACC (Chi-square test, p=0.025 and p=0.0016, respectively), being negative in all cases, as compared to conventional and myxoid variants. p-mTOR was also negative in tumors with high Weiss Score (Chi-square test, p=0.025), whereas SSTR3 was negative in tumors with low Weiss Score (Chi-square test, p=0.01). Finally, SSTR1 was associated to cases having a high mitotic activity (Chi-square test, p=0.007).

Univariate analysis showed that high mitotic rate (p=0.0132), high Ki-67 proliferation index (p=0.0001) and SSTR5 expression (p=0.0173) were associated with shorter overall survival (**Table 3)**. At multivariate analysis, Ki-67 and SSTR5 expression remained significant independent parameters (p=0.004 and p= 0.024, respectively).

DISCUSSION

In this study, we demonstrated that pasireotide and everolimus interact differently when combined with mitotane or with each other, determining variable effects on viability of ACC cell lines. Moreover, SSTR5 expression was found to be significantly associated with a poor prognosis in a cohort of resected ACCs.

ACC cell lines. As the most frequent molecular abnormality in ACC affects the IGF axis, which ultimately leads to an activation of the mTOR pathway, several studies investigated the effectiveness of mTOR inhibitors in ACC cell lines. Consistently, an antiproliferative effect was observed when used as a single agent [14] or in combination, either with a tyrosine kinase inhibitor (everolimus *plus* sorafenib) [16] or mitotane (temsirolimus *plus* mitotane). This paper aimed to investigate *in vitro* the potential synergistic effects of the mTOR inhibitor everolimus

and/or the somatostatin analog pasireotide with mitotane. We found that everolimus induced a significant inhibition of cell growth in both mitotane sensitive and resistant cell lines, but its effect was reduced when administrated in combination with mitotane. Conversely, the association of pasireotide with mitotane or everolimus determined opposite effects in H295R and SW-13 cell lines. We previously demonstrated that in H295R cells, the association of effective drugs, namely gemcitabine and mitotane, caused a reduction of citotoxic activity of these compounds on cell viability [31]. Similarly, in the present study, all combinations of drugs produced antagonistic effects on the inhibition of growth in such cell line. Interestingly, in mitotane resistant SW13 cells, the combination of pasireotide with mitotane, both determining poor effects on cell viability when administrated as single agents, was synergistic, though not effective enough to generate a significant growth inhibition. Conversely, the combination of mitotane and everolimus in the same mitotane resistant cell line determined an antagonistic effect, despite everolimus efficacy when administered alone. Anyway, in both cell lines, the synergy or antagonism caused by drug combinations was unable to induce relevant changes on cell viability compared to single treatments. In H295R cells, the insufficient or reduced effects of pasireotide, alone or in combination with mitotane and everolimus, went along with an up-regulation of all somatostatin receptor subtypes, an event that did not occur in SW13 cells. These findings indicate that H295R and SW13 cells had different response profiles to the combined or single drug treatments, and that their mechanism(s) of action involved different pathways. Although our *in vitro* data are opposite to those reported by De Martino et al. [13] with temsirolimus and mitotane on the same cell lines, they support the clinical experience of Fraenkel et al. [12], who reported the failure of everolimus to induce any therapeutic benefit in advanced ACC patients, who were heavily pretreated with multiple lines of systemic agents, including mitotane. CYP3A4, which metabolizes everolimus, is strongly induced by mitotane, and this effect persists up to one year after

interruption of mitotane treatment [32, 33]. Therefore, interactions between mTOR and mitotane are common and could be expected [33]. The drug interaction induced by the combination of targeted molecular agents with adjuvant mitotane can potentially result in reduced therapeutic efficacy of these compounds in the treatment of ACC. Therefore the therapeutic efficacy of everolimus should be evaluated prior to treatment with mitotane and monitoring of the blood levels of these drugs will be essential in assuring their putative effect.

ACC tissue. Searching for other explanations of the treatment failure and to verify a potential differential protein expression in ACC according to clinico-pathological features, we investigated the expression of SSTR and of phosphorylated molecules of the mTOR pathway by means of immunohistochemistry in 58 ACC cases. Our data are consistent with the previously described variability of SSTR expression in ACC samples [17, 19]. We found that the absence of SSTR3 was correlated with the oncocytic variant, which is the least aggressive variant of ACC, and with low Weiss Score tumors. Interestingly, Mariniello et al. [19] reported the absence of SSTR3 in normal adrenal and in the majority of adrenocortical adenomas. Moreover, we observed a higher expression of SSTR1 in cases with high mitotic rate and a relationship of SSTR5 expression with a worse outcome. Although an antisecretive effect of pasireotide on ACC cells has been reported *in vitro*, we did not identify any correlation with the functionality of the tumor. Concerning mTOR pathway molecules, they were variably expressed, but unlike De Martino et al. [34], we could not confirm a reciprocal correlation between the expression levels of the phosphorylated form of mTOR and either S6K or 4EBP. A specific signature predicting response to mTOR inhibition has not been identified, yet. De Martino et al. [34] reported that at least a subgroup of the tumors with more aggressive pathological phenotype (high Weiss Score), showed weak staining of p-S6K and p-4EBP, indicating that a subset of less differentiated ACCs could be less dependent on the

activation of the mTOR pathway. The identification of the phosphorylated form of mTOR, S6K and 4EBP, cannot be employed as an established surrogate marker of mTOR inhibitor efficacy. However, the immunohistochemical overexpression of SSTR and mTOR pathway molecules can suggest the activation of these targets, at least in a subset of patients.

In conclusion, all combinations among mitotane, pasireotide and everolimus produced an antagonistic effects on mitotane-induced growth inhibition in the mitotane sensitive H295R cell line. This suggests a prudent clinical approach in designing therapeutic combinations of mitotane with other drugs potentially active in ACC, such as mTOR inhibitors or somatostatin analogs. An heterogeneous expression profile of mTOR-related molecules and SSTR(s) was observed in ACC samples, thus confirming that only a subset of patients with ACC might be more sensitive than other and benefit from treatment with these drugs. Further studies are required to better refine the potential biomarkers predictive of response to mitotane, mTOR inhibitors or somatostatin analog treatment(s) in adrecortical cancer.

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FIGURE LEGENDS.

Figure 1. Cell viability after single or combined pasireotide, mitotane and everolimus treatment. Cytotoxic response to pasireotide, mitotane and everolimus and their combination in H295R and SW13 cell lines. Data result from three different experiments (±SD, n=3) and are expressed as ratios of proliferating cells, as compared to basal conditions.

Figure 2. SSTR subtype gene expression after single or combined pasireotide, mitotane and everolimus treatment. SSTRs gene expression analysis in H295R and SW13 cell lines under different conditions. Data are expressed as fold changes ($2^{-\Delta\Delta Ct}$). A fold change >2 was considered significant.

Figure 3. Representative examples of ACCs immunostained for p-mTOR (A), p-70S6K (B), p-AKT (C), p-4EBP (D), p-RAPTOR (E), SSTR1 (F), SSTR2 (G), SSTR3 (H), SSTR4 (I), SSTR5 (L). (A-L: immunoperoxidase, original magnification 400X).